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A STUDY OF THE MECHANISM OF ACTION AND ROLE OF A NATURAL INHIBITOR OF DOPA AUTOXIDATION ISOLATED FROM GUINEA PIG SKIN*

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ABSTRACT

The naturally occurring inhibitor of DOPA autoxidation present in guinea pig skin has been shown to inhibit the oxidation of tyrosine to melanin by both the enzyme tyrosinase and epidermal melanocytes.

Two possible modes of action were investigated and the inhibitor was shown to be effective by competing with the enzyme tyrosinase for the substrate. ^{14}C-labeled tyrosine became irreversibly bound to the inhibitor, thus prevented from further oxidation. Acid hydrolysis of the inhibitor-substrate complex released the tyrosine, which had not been changed.

During the last thirty years considerable interest has been shown in the inhibitor of DL-3,4-dihydroxyphenylalanine (dopa) autoxidation which is extractable from many mammalian tissues, although skin and melanoma cells have provided its major source. Most workers have concentrated on characterizing the inhibitor and there are many conflicting views on this subject. These have been discussed in the preceding paper, which also presents our own finding (1). The materials described can be broadly divided into two main categories—those described as being heat-stable, dialyzable, non-protein, sulfhydryl compounds and those which are non-dialyzable, non-sulfhydryl, probably proteinaceous compounds.

Since the inhibitor is found in the skin along with the melanin synthesizing system, and since the biosynthesis of melanin in epidermal melanocytes is believed to involve the enzymic oxidation of dopa as its second biochemical stage, it is not difficult to understand why a role has been postulated for this inhibitor in the normal control of pigmentary levels in skin.

The dopa autoxidation system has been used routinely as an assay for the study of this inhibitor, but apart from the study of Hirsch (2), which demonstrated the action of the inhibitor in intact cells, little is known of the relevance of the inhibitor to the control of normal melanin biosynthesis in skin or its ability to inhibit tyrosinase catalyzed oxidation of tyrosine *in vitro*.

Our own investigations using the dopa autoxidation system which has been used widely during the study of the inhibitor have indicated that the active factor is a protein of molecular weight about 6000, which requires the integrity of its spatial structure in order to function; it is not dependent upon the presence of sulfydryl groups, and is heat- and U.V.-stable (1).

The present study was designed to answer several questions: Is the inhibitor able to prevent the oxidation of tyrosine to melanin by the enzyme tyrosinase? If so, how? Does the inhibitor have the ability to reduce melanin biosynthesis by melanocytes?

MATERIALS AND METHODS

The inhibitor of dopa autoxidation was extracted from white guinea pig skin, and purified as described previously (1). The inhibitory capacity of each preparation was assayed by the serial dilution technique (1) and ascribed a number of units of activity.

Melanin synthesis by tyrosinase. The assay of tyrosinase activity was performed in three matched silica cells. One contained 1 ml 0.5M phosphate buffer pH 6.5, 1 ml 0.001M tyrosine in water and 1 ml water, the second contained 1 ml buffer, 1 ml tyrosine and 0.9 ml water and the third contained 1 ml buffer, 1 ml tyrosine and 0.8 ml water. The three mixtures were oxygenated for 5 minutes, and then 0.1 ml tyrosinase solution (mushroom tyrosinase; Koch-Light Laboratories Ltd., Colnbrook, England, 50 enzyme units per ml water) was added to the last two cells; 0.01 ml inhibitor was also

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TABLE I

Composition of solution used for the study of the mechanism of inhibition

	A	B	C
0.5M phosphate buffer pH 6.5	3.00 ml	3.00 ml	3.00 ml
3×10^{-3}M tyrosine in water	3.00 ml	3.00 ml	3.00 ml
C^{14}-tyrosine (25mc/ml)	0.05 ml	0.05 ml	0.05 ml
Water		3.00 ml	1.50 ml
Inhibitor	3.00 ml		3.00 ml
Tyrosinase (50 units/ml)	1.50 ml	1.50 ml	
	10.55 ml	10.55 ml	10.55 ml

added to the last cell. The contents were mixed, and the absorbancies of each cell were measured at 280 mμ every minute for 35 minutes.

Melanin synthesis by epidermal melanocytes. A black-skinned guinea pig was sacrificed by carbon dioxide asphyxiation. Its hair was removed by preliminary treatment with electric clippers, followed by epilating wax. The whole skin was removed and held taut between two clamps while slices consisting of equal thicknesses of dermis and epidermis were taken with a dermatome. The slices were cut into squares measuring about 1 cm and the epidermis removed by incubating in either 0.5% trypsin in Ringers solution at 37°C for 2 hours or 2M sodium bromide in water at room temperature for 3½ hours. After this time the sheets of epidermis could be removed with gentle use of scalpel and forceps. The epidermal sheets were washed thoroughly in Ringers solution, and then floated on culture medium in petri dishes. The medium consisted of 199 tissue culture medium (Burroughs Wellcome & Co., Beckenham, England) supplemented with 10% calf serum and 0.01 ml of a solution containing 0.1 mc/ml universally labeled C^{14}-tyrosine hydrochloride (CFB 74, Radiochemical Centre, Amersham, England). Inhibitor preparation, or water as control, was added at the rate of 1 ml to 4 ml medium.

The cultures were incubated at 37°C for 48 hours. After incubation the tissues were blotted, weighed, and transferred to screw-capped tubes with 4 ml 6N hydrochloric acid. The tissues were hydrolyzed at 100°C for 24 hours. Electron microscope studies had previously shown that this treatment rendered soluble all camponents of the tissues except for the melanin, which was harvested on Millipore filters, and thoroughly washed with water. After drying, the filters were dissolved in scintillant fluid which consisted of 6 g PPO and 250 ml cellosolve. Cab-O-Sil was added to the scintillant to form a gel which maintained the melanin in an even suspension. The radioactivity was measured in a Packard Tricarb liquid scintillation spectrometer.

The filtrate and washings were pooled, diluted to 20 ml and a portion assayed for radioactivity. The filtrate was considered to contain all the protein of the epidermis, and the radioactivity would give a measure of protein synthesis during culture.

Study of mechanism of inhibition. The fate of tyrosine when incubated with tyrosinase in the presence and absence of the inhibitor was likely to give a clue to the mechanism of the inhibition. Two hypotheses were proposed, one suggesting that the tyrosine was converted enzymatically by the inhibitor to a small molecular weight material which was an unsuitable substrate for tyrosinase, the second that the tyrosine was bound by the inhibitor, in such a way as to be unavailable to tyrosinase.

Three mixtures were prepared, as in Table I. These were incubated at 37°C for 24 hours in a shaking water bath. After incubation each mixture was subjected to:

1) Thin-layer chromatography and autoradiography. 10 μl samples of each incubation mixture and standards were applied to thin-layer plates of Silica Gel G, and allowed to dry. The chromatograms were developed in n-butanol-acetic acid-water, 80:20:20 v/v. After development the chromatograms were sprayed first with 0.05% potassium ferricyanide in 0.2M acetate buffer pH 6.0 to locate dopa and then with 0.5% ninhydrin in acetone to locate tyrosine and other amino acids. The color of the spots was intensified by heating to 100°C for a few minutes.

Autoradiographs were produced by exposing a sheet of dental x-ray film to each thin-layer plate for one week in the dark. The films were developed for 6 minutes in Dental X-ray developer (phenidone-hydroquinone) diluted 1:3 with water. They were then washed in water and fixed for 2 minutes in Hypam fixer, diluted 1:4 with water. The film was rinsed thoroughly in running water and dried in the air.

The radioactivity detected by the film was related back to the thin-layer plates. Each radioactive area was then scraped into a scintillation vial, suspended in scintillant liquid and Cab-O-Sil, and the radioactive content assayed.

2) Gel-filtration. A column of Biogel P10 (0.75 × 25 cm) was loaded with 1 ml samples of the incubation mixtures, which were then eluted with water. The eluant was monitored at 280 mμ for protein concentration, and 1 ml fractions collected on a Gilson automatic fraction collector.

0.2 ml from each fraction was mixed with 10 ml scintillant liquid and the radioactive content assayed.

RESULTS

The oxidation of tyrosine by tyrosinase and the effect of the inhibitor of dopa autoxidation on this system is shown in Figure 1. The reaction was measured by an increase in absorbancy as tyrosine was converted to the quinone.

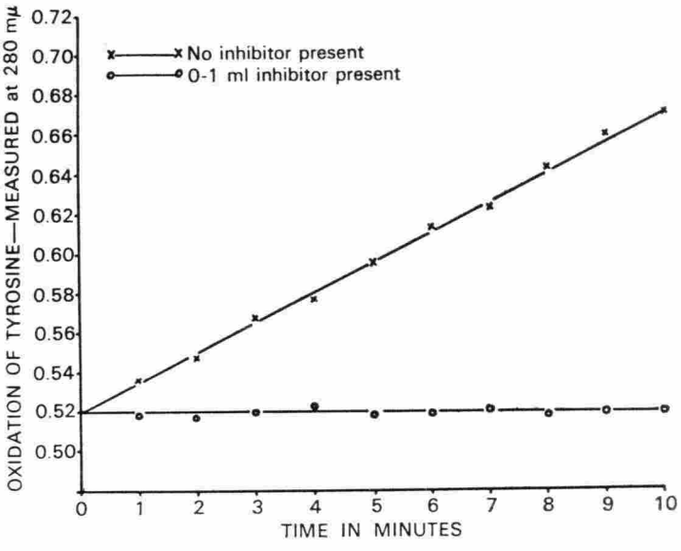

FIG. 1

The oxidation was found to be proportional to the enzyme concentration and was linear during the 15–20 minutes after the initial lag phase of 15 minutes. The lag phase is not shown in Figure 1. The presence of 0.1 ml dopa autoxidation inhibitor caused complete inhibition of this enzyme-controlled reaction. This result gave more significance to the possibility that the inhibitor is involved in the control of pigment production in the skin, since tyrosine oxidation by tyrosinase is the start of the normal metabolic route to melanin.

The next step was to measure the effect of the inhibitor on melanin synthesis by the skin. Table II shows typical amounts of protein and melanin synthesized by sheets of epidermis prepared by both sodium bromide and trypsin splitting, and the effect of the inhibitor upon these.

The method of preparation of the epidermis caused little variation in the amount of melanin synthesized, although protein synthesis was very much lower in sheets prepared with sodium bromide, than in those prepared by trypsin.

Sodium bromide produced epidermal sheets more easily than trypsin, and although the tissue is not normally considered to be viable following this treatment, some protein synthesis is believed to have occurred. Histological examination showed the tissue to be slightly degenerate after sodium bromide treatment although the melanocytes appeared to be intact and functioning normally. Trypsination is an accepted method of separating dermis and epidermis, and the cells will grow in tissue culture after such treatment, but it is difficult to obtain epidermis in large sheets as it tends to fragment.

The inhibitor of dopa autoxidation caused a marked decrease in melanin synthesis in epidermis prepared either way, about 65% of that in the control.

Having presumptive evidence that the factor has an inhibitory effect on melanin synthesis in melanocytes the next step was to investigate its mode of inhibition.

Mode of inhibition. After 24 hours of incubation, the three mixtures, A (with both tyrosinase and inhibitor), B (tyrosinase alone) and C (inhibitor alone), showed considerable differences. A had a clear, very pale brown appearance, B had a dense, black precipitate, and C was water clear with no precipitate.

1) Thin layer chromatography and autoradiography revealed that no small molecular weight material had been produced which was separated from tyrosine by the solvent system chosen. It was therefore concluded that the inhibitor did not exert an enzymic effect on the tyrosine, and it had not been diverted along a path away from that of melanin biosynthesis.

In each case radioactive tyrosine was found to be present, and a quantity of radioactivity was recoverable from the origin spot. Subsequent thin layer chromatography showed that both melanin and the inhibitor are unmoved by this solvent system and it was therefore

TABLE II

The effect of the dopa autoxidation inhibitor on melanin biosynthesis by pieces of epidermis prepared by two methods

	Protein synthesized	Melanin synthesised
	expressed as DPM per g tissue	
Epidermal sheets prepared by trypsination		
Control	191,000	147,000
With inhibitor	187,000	96,000
Epidermal sheets prepared by sodium bromide		
Control	53,000	146,000
With inhibitor	54,000	99,000

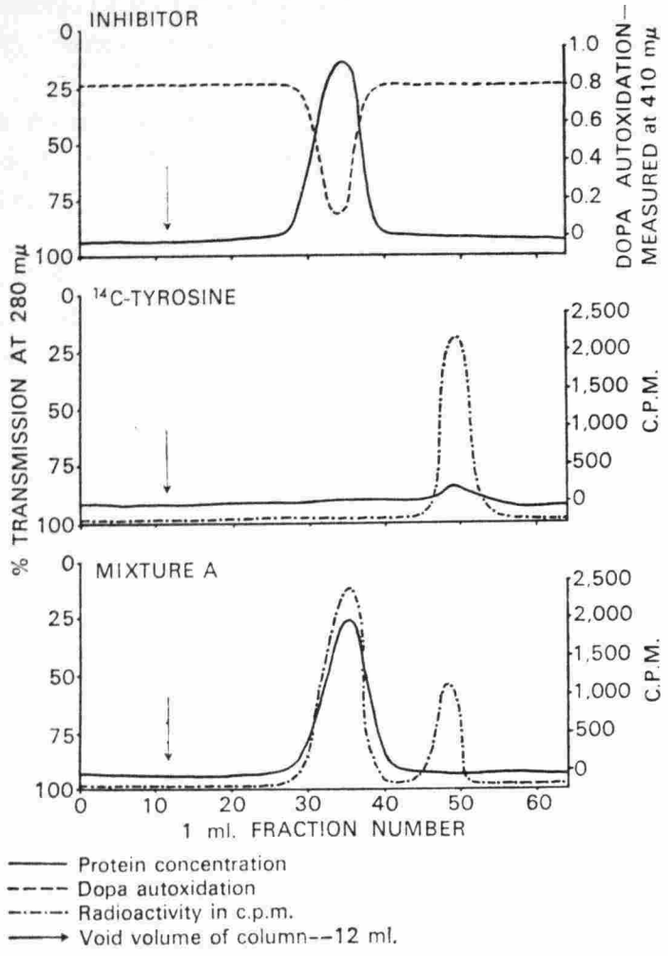

FIG. 2

impossible to determine which had contributed to the activity recoverable in this area.

2) Figure 2 shows the locations of the inhibitor and tyrosine respectively on elution from a column of Biogel P 10. These are clearly separable. Figure 2 also shows an identical Biogel separation of Mixture A. Only one protein peak is detected and this corresponded to the position of the inhibitor. Two radioactive peaks were present however; one is certainly tyrosine, while the other was superimposed on the protein peak associated with the inhibitor. Mixture C shows protein and radioactive peaks similar to those of A.

Fractionation of mixture B showed a distribution of radioactivity similar to that obtained when active tyrosine alone was passed through the column. Melanin remained on the top of the gel and tyrosinase was of course totally excluded and emerged in the column void volume.

These results indicate that the inhibitor and tyrosine bind together to form a complex, so that the substrate is no longer available for tyrosinase action.

DISCUSSION

Many workers have studied the structure of the natural inhibitors of dopa autoxidation found in animal tissues and the general consensus of opinion points to two types being extractable, one having a relatively low molecular weight and one having a high molecular weight. The one described in this paper is of the latter type, and was the only one found to be present in our extracts, and it was distributed widely throughout the organs of the guinea pig.

It was likely that it might be functioning in its capacity of dopa autoxidation inhibition simply as a reducing agent, e.g. cysteine or glutathione (3) have been implicated by some workers, both in this context and in that of the normal control of pigmentary levels in skin, but this can now be ruled out in the present case.

Tyrosine is utilized in several biochemical systems e.g. for the synthesis of such small molecules as p-hydroxyphenyl pyruvic acid, but the hypothesis that the inhibitor is an enzyme capable of rapidly converting tyrosine to one of these is not established by the thin layer chromatographic evidence presented here. However it was remotely possible that the product of such a conversion might have a similar R_F value to tyrosine in the solvent system described, and so other solvents were tried without evidence of resolution of other radioactive materials.

We have shown here that the inhibitor is not only capable of causing inhibition of dopa autoxidation but has a marked effect on melanin synthesis from tyrosine both by tryosinase directly and by living melanocytes. Since the inhibitor is extracted from the skin where it must exist near the site of tyrosinase, it is likely that it plays a significant role in the control of the level of pigment in the skin.

Studies on its mode of action have led us to conclude that it inhibits by binding to the substrate, which may be dopa or tyrosine, so that the latter is no longer free for oxidation by tyrosinase.

In previous studies little has been reported on the mode of action. Those reports which are available agree in principle with the results described here.

Cooper and Mishima (4) have described their work on a heat-labile inhibitor isolated from malignant melanomas. This inhibitor had a molecular weight below 10,000 and combined with the substrate, tyrosine, thus preventing its conversion to melanin.

Karkhanis (5) found that mushroom tyrosinase had a protein inhibitor associated with it, right up to the last stage of purification. Further examination revealed that the inhibitor was a protein of molecular weight 100,000. It was an apoenzyme which combined with a copper ion as a prosthetic group and formed fully active tyrosinase enzymes. Keilin and Mann (6) suggested that tyrosinase existed in the skin in an inactive form and took up copper to activate itself when necessary. While in the inactive form the molecule was still capable of binding to the substrate but was unable to metabolize it.

Seiji *et al.* (7) believe that the inhibitor is a small non-protein sulfhydryl molecule, and have tentatively identified it as glutathione. They have studied the mode of inhibition by glutathione and have found that it binds, not to the substrate, but to one of the oxidation products of the substrate, thus preventing its further oxidation to melanin. They have not positively identified the complex but they believe it to consist of glutathione and dopa quinone.

Hirsch holds firmly to the idea that the inhibitor acts by binding tightly to the copper or other metal ions which are necessary for catalyzing dopa autoxidation (8).

In spite of the wide disagreement which exists on the actual structure of the inhibitor, most workers seem to agree that its mode of action is one of competitive inhibition with the enzyme tyrosinase. Either the substrate or one of its immediate oxidation products binds to the inhibitor so that oxidation to melanin does not occur.

Not only is this significant in the control of normal skin pigmentation, but Demopoulos (9) has reported the selective inhibition and regression of malignant melanomas by tyrosine deprivation.

The role of this material in skin pigmentation control has not been resolved, since our own tentative attempts to produce skin lightening in live animals by both topical and parental administration of the inhibitor have been unsuccessful.

REFERENCES

1. Flawn, P. C. and Wilde, P. F.: Isolation and partial characterisation of the natural inhibitor of dopa anutoxidation in skin. In press.
2. Hirsch, H. M.: Tissue autoxidation inhibitors. II. The presence of inhibitor in intact cells; assay of liver and hepatoma; effect on radio oxidations. Cancer Res., *16:* 1076, 1956.
3. Flesch, P. and Rothman, S.: Role of sulfhydryl compounds in pigmentation. Science, *108:* 505, 1948.
4. Cooper, M. and Mishima, Y.: Substrate limiting melanogenic inhibitor in malignant melanomas. Nature, *216:* 189, 1967.
5. Karhanis, Y. D.: The inhibition of mushroom tyrosinase by its apoenzyme, a protein inhibitor, and certain reagents. Ph.D. Thesis, Florida State University, 1961.
6. Keilin, D. and Mann, T.: Polyphenol oxidase—purification, nature and properties. Proc. Roy. Soc., *125B:* 187, 1938.
7. Seiji, M., Yoshida, T., Itakura, H. and Irimajiri, T.: Inhibition of melanin formation by sulfhydryl compounds. J. Invest. Derm., *52:* 280, 1969.
8. Hirsch, H. M.: Tissue autoxidation inhibitors. I. The inhibition of dopa autoxidation by extracts from normal and neoplastic tissues. Cancer Res., *15:* 249, 1955.
9. Demopoulos, H. B.: Effects of reducing the phenylalanine-tyrosine intake of patients with advanced malignant melanoma. Cancer, *19:* 657, 1966.